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THE MICROSCOPIC STUDY OF BACTERIA AND FUNGI IN SOIL.

H. JOEL CONN.

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H. JOEL CONN.

SUMMARY.

1. A method of staining dried soil infusions has been devised, similar to the one already in use for milk, by means of which direct microscopic study of the bacteria in soil is possible.

2. The method has certain limitations, arising primarily from the fact that it is difficult to distinguish between the smallest soil bacteria and tiny particles of dead organic matter; but in spite of these limitations it has proved of use, first to give an idea as to the actual number of bacteria in soil, and second to furnish information as to the kinds of microörganisms that are present in soil in active form.

3. The microscope shows that the actual number of bacteria in soil is probably five, ten, or even twenty times as great as indicated by the culture plate method. The discrepancy between plate count and microscopic count is due to bacteria that do not grow on the plates rather than to the occurrence of large clumps that do not break up in the process of plating. This is shown by the fact that a large majority of soil bacteria occur singly.

4. The microscope furnishes additional evidence in confirmation of one point brought out in previous papers: namely, that the large spore-formimg bacteria (***Bacillus megatherium*** DeBary and ***B. cereus*** Frankland, for instance), which are abundant on culture plates made from soil, actually occur in normal soil only as spores. These spores, moreover, form a very small proportion of the total bacterial flora of soil.

5. The method has not revealed the presence of fungus mycelium in any soil except where there is an unusual amount of organic matter, such as in the leaf-mold of woodland soil.

6. Filaments of Actinomycetes have been found, altho in much smaller numbers than the spores of these organisms.

7. In the case of these two groups of organisms, fungi and Actinomycetes, the plate count is not an index of activity, but of ability to produce spores. In general it may be said that when an organism is concerned that produces spores or any other resting stage the significance of the plate count cannot be determined unless the microscope is used to show whether the organism is present in active form.

INTRODUCTION.

The methods in general use for the study of soil bacteria are all cultural methods. The culture plate is used to estimate their numbers; pure culture study of the organisms isolated from soil is used

to reveal their individual activities; while the inoculation of sterile culture media with soil is the method used when it is desired to study mass actions of the soil bacteria. Much as these methods differ from each other, they are all cultural and therefore indirect. Cultural methods show what takes place under laboratory conditions, but furnish no direct evidence as to what occurs in nature.

In the study of pathogenic bacteria — either animal or plant parasites — there has never been any such entire dependence upon indirect evidence. Even before cultural methods of studying bacteria were devised, microscopic examination of infected tissue was common. Microscopic study alone did not solve the problems of infection; but its importance has always been so evident that even after the necessity of studying bacteria in pure culture was realized, the use of the microscope was not abandoned. Non-pathogenic bacteria, to be sure, have often been counted by means of the microscope, as recently discussed by Breed and Brew in a bulletin of this Station,[1] and in enumerating the bacteria in milk, the microscope is now used quite extensively. It is also used to some extent as a means of counting bacteria in water and in various foods. Except in pathogenic work, however, few bacteriologists have realized the full possibilities of the microscope.

The greatest limitation of the microscope in counting minute objects is well summed up by Breed and Brew, as follows: "One of the chief difficulties which is met with in using the microscope in this way has been the preparation of the substance containing the bacteria, yeasts, blood cells, and the like, in such a way as to show the objects to be counted distinctly and at the same time evenly distributed over definite areas."[2] These difficulties are harder to overcome in the case of soil than in the case of milk — to which Breed and Brew applied the microscopic method. The prospects of applying the method to soil appeared at first quite discouraging, because the ordinary bacterial stains color not only the microörganisms but also the dead organic matter and even the mineral matter in soil. By the use of the proper technic, however, as already pointed out,[3] this difficulty has been overcome, and it is now possible to make a rough estimate by means of the microscope as to the actual number of bacteria in any soil.

Quantitative estimations, however, are not the only possibilities of the microscope in studying a bacterial habitat. The successful use of the microscope in counting milk bacteria has overemphasized the quantitative side of the matter; and it must not be forgotten that another side exists. Pathologists have amply demonstrated this in their use of the microscope for the study of infected tissues.

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[1] Breed, R. S., and Brew, J. D. Counting bacteria by means of the microscope. N. Y. Agr. Exp. Sta., Tech. Bul. 49. 1916.

[2] Page 5 of reference given in footnote 1.

[3] Conn, H. J. The direct microscopic examination of bacteria in soil. *Abstr. Bact.*, 1:40. 1917.

The purpose of the present paper is to show to what uses the microscope may be put in the study of soil bacteria, qualitatively as well as quantitatively.

TECHNIC.

Preparation of soil infusion.— The first step in making microscopic preparations to show the bacteria in soil is to prepare a soil infusion that is capable of adhering evenly to a glass slide and that contains the solid matter in suspension in such a strength as not to be too opaque and yet not too dilute to show the bacteria in sufficient abundance for study. Pure water does not yield a satisfactory infusion, because it cannot be spread evenly over glass unless the glass is absolutely free from grease, and also because there is not sufficient organic matter in soil to fix the infusion to the slide upon drying. After trying various fixatives, a weak solution of gelatin has been found best, other adhesive substances such as albumin having been found to have too great affinity for the stains used.

The gelatin fixative is prepared by dissolving 0.15 g. of gelatin in 1000 c. c. of hot water. It is recommended that some gelatin be used which is put up in granular form, because of the greater ease in measuring out such a small quantity. The exact strength of the gelatin solution is quite important; if too strong it coats the soil granules with a film that stains the same color as the bacteria, while if too weak it will not adhere properly to the glass. The solution should be sterilized in a cotton-plugged flask or bottle and resterilized after each day that it has been in use. A new solution should be made up whenever the old solution is suspected to be no longer satisfactory.

The extent to which to dilute the soil in making the infusion depends primarily upon the amount of clay it contains. The infusion should be as concentrated as possible without causing the preparation to be too opaque for satisfactory study. For clay soil, which presents the greatest difficulties, a dilution of 1/10 has proved the best. Sandy soil is much more readily studied, and a greater concentration may be used, such as 1/3 or 1/5. In making the infusion, add one gram of soil to the proper amount of the gelatin solution, allowing 0.5 c. c. for the volume of the 1 g. of soil. That is, to make a dilution of 1/10, add 1 g. of soil to 9.5 c. c. of the fixative. Small glass-stoppered weighing bottles have proved very convenient for making the infusions. The soil and the fixative should be mixed in the bottle, shaken for about a minute, and the portion for examination withdrawn before the sediment has had time to settle. The glassware used should be clean, but not necessarily sterile.

Preparation of smears.— Each smear should be made from 0.1 c. c. of the infusion, measured out with a capillary pipette. A convenient form of pipette is made out of thermometer tubing with a white background, calibrated to deliver from one mark to a second mark. A pipette of much this style is furnished with Hayem-

Sahli blood counting outfits for measuring out white corpuscles, and can be used by letting the infusion run out from the 15 to the 5 cu. mm. mark. If accurate quantitative results are desired the calibration of the pipette should be tested before use.

The microscopic slides should be well cleaned and rinsed in alcohol just before using, or the infusion will not adhere properly. It is recommended that two smears be made on each slide. Each smear should be made to cover 1 sq. cm., obtaining this size by placing the drop of infusion over a sheet or plate on which square centimeter areas are drawn and spreading it out with a needle. Care must be taken to see that the visible soil particles are evenly distributed .thruout the entire area. As soon as the smears are made the slides should be placed on a level surface over a water-bath of boiling water, where they will quickly dry. They are then ready for staining.

Staining.— The stain used is rose Bengal in carbolic acid.[4] To prepare, add 1 g. of the dye to 100 c. c. of 5 per ct. phenol. This stain can be kept two or three months without deterioration. A drop of the stain should be placed upon the smear while the slide is still resting on the water bath, and allowed to remain for one minute. Enough stain should be used to prevent drying during the minute of heating. At the end of the minute wash the slide very briefly by immersion in water, without allowing it to stay in the water an appreciable length of time.[5]

After drying, examine under a microscope to see if the preparation is good. A good preparation should show the bacteria a deep pink or red, the mineral particles uncolored, some of the dead organic matter light pink but most of it either yellow or unstained. If the bacteria are faintly stained or if everything is stained pink,

[4] Two other dyes of the phthalein series have been tested, namely eosine and phloxine. Neither gives permanent results. A 5 per ct. solution of phloxine in 5 per ct. phenol gives very good temporary results, altho the preparations fade rapidly. Good results have been obtained occasionally with eosine, but the technic is more difficult and the results less dependable.

[5] In the abstract of the previous paper on this subject (see footnote 3) a slightly different technic was mentioned. Two different staining methods were given, one of which called for rose Bengal or phloxine and differed from the above only in detail. Later work has shown the superiority of rose Bengal over phloxine and has shown the above technic to be an improvement over that previously given. The other method of staining (the fuchsin-methylen-blue method) has been found to give unsatisfactory results for research work. The preparations are very striking, however, for demonstration purposes, so perhaps the technic should be described. It is as follows:

To 40 c. c. water add 3 c. c. saturated alcoholic solution of methylen-blue and 0.8 c. c. saturated alcoholic fuchsin (each measured accurately). Immerse the slide bearing the dried soil infusion into this stain and allow it to remain from one to three minutes. Wash in water, dry and examine. The bacteria should be red, mineral particles blue, and organic matter blue to purple. The deeply stained soil particles so often obscure the bacteria that only a small portion of the total flora is visible, and if the method is used for demonstration purposes, recently manured soil or other soil with a rich bacterial flora should be used.

a new preparation should be made. The former condition generally indicates too long washing, the latter too great concentration of gelatin in the fixative or too old stain. Faint staining is especially troublesome, for it is almost sure to cause some of the bacteria to be overlooked. Whenever a count is suspiciously low and the bacteria do not stand out very plainly it is better to wash off the immersion oil in xylol and restain, or still better to make a new preparation, provided the trouble is discovered soon enough.

Microscopic examination.— These preparations should be examined with an oil-immersion objective and a high-power eye-piece. The combination of lenses found to be most satisfactory is a 1.9 mm. (1/12 inch) fluorite objective (having a numerical aperture of 1.32) with a 12.5× ocular. An Huygenian ocular of this magnification can be used; but with a compensating ocular it has been found possible to obtain a greater depth of focus. When counting bacteria it is advisable not to count the entire field, but to have the central portion of it marked off as described by Breed and Brew for milk work.[6] They used a disc in the eye-piece with circles and cross-lines ruled on it so as to limit the area within which the bacteria were counted. A similar arrangement may be used for soil, the size of the circle on the disc depending upon whether an Huygenian or compensating ocular is used. It has been found convenient to use a circle of such a size as to cover an area on the slide either 80 microns or 113 microns in diameter. Every organism in the former sized area represents approximately 2,000,000 per cubic centimeter of the soil infusion;[7] every organism in the latter 1,000,000 per c. c.

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[6] Page 26 of reference given in footnote 1.

[7] This is computed as follows:

Let r stand for radius of circle.

Then $r = \frac{80}{2}$ micron = 40 micron = 0.04 mm.

$r^2 = 0.0016$ sq. mm.

Area of circle = $\pi r^2 = 3.1416 \times 0.0016$ sq. mm. = 0.005027 sq. mm.

$$\frac{\text{Area of smear}}{\text{Area of cirle}} = \frac{1 \text{ sq. cm.}}{0.005027 \text{ sq. mm.}} = \frac{100 \text{ sq. mm.}}{0.005027 \text{ sq. mm.}}$$

= 19,900 or approximately 20,000.

Therefore each circle = approx. $\frac{1}{20,000}$ of entire smear.

But the smear contains only 0.01 c. c. of soil infusion.

Hence each circle contains approx. $\frac{1}{20,000} \times 0.01$ c. c.

$= \frac{1}{2,000,000}$ c. c. of soil infusion.

The computation for the larger size field is made in exactly the same way, in this case using the value:

r = 0.0565 mm.

(If soil has been diluted ten times, these factors should be multiplied by 10; i. e. 20,000,000 and 10,000,000 per gram of soil). It is possible to get the circle used to cover an area of either of these sizes by adjusting the length of the draw-tube; but it is better to use a circle of such a size as to give an even factor with the draw-tube down, as the most satisfactory results are obtained with a tube-length of 160 mm.[8]

It is important that a good source of illumination be used in this work. The brightest daylight can be used; but a better and more constant source of light can be obtained by artificial means. An electric light filtered thru " daylight " glass is very satisfactory; or a welsbach light filtered and condensed thru a blue globe containing ammoniacal copper sulphate. Minute organisms may easily be overlooked if the light is not good.

One word of caution is not out of place, altho to anyone experienced with a microscope it is unnecessary. As the smear of soil infusion is of too uneven thickness to allow the use of a coverglass, there is always danger that the objective will come into contact with the dried soil. Under such circumstances a slight motion of the slide might easily scratch the lens; so it is best to make it a rule never to move the slide unless the objective is at least far enough away from it to bring the objects in the smear into focus. With this reasonable care, no noticeable injury has been caused to an objective used extensively in this work for nearly two years.

LIMITATIONS OF THE METHOD.

In order to interpret correctly the results obtained it is necessary to recognize the limitations of the microscope in this work. These limitations may be considered under two heads: (1) those that apply in quantitative work alone, and (2) those that must be taken into account when using the method for qualitative purposes.

In quantitative work.— As already discussed there are certain limitations inherent in any microscopic method of counting bacteria. They are due to the difficulties in spreading the material evenly over the slide and in distinguishing the bacteria from other objects. Both of these difficulties are especially hard to overcome when the method is used for studying soil. Even distribution of the material is harder to accomplish for a solid like soil than for liquids like milk, blood or culture fluids. Also, the difficulty of distinguishing bacteria

[8] In the present work the circle on the disc in the ocular measured exactly 1 cm. in diameter. Used in connection with Bausch and Lomb 12.5× compensating ocular and 1.9 mm. objective this was found to cover an area of such a size as to give a factor of 1,000,000 per c. c. of soil infusion with the draw tube of the microscope down. As the slightest variation in the size of the circle would cause considerable variation in the size of the area included within it, the area should always be measured with the aid of a stage micrometer, even tho exactly this combination of lenses be used.

is increased in the case of soil, because of the abundance of tiny clay particles or fragments of organic matter that closely resemble bacteria.

The first of these difficulties — uneven distribution — may be partly overcome by examining a number of different smears from the same sample. It is impossible, however, to prevent bacteria from lying under soil particles or masses of organic matter; and such material generally obscures the bacteria because of its high refractive power even in well-stained preparations, in which it should be uncolored. Sometimes such a dense field is encountered that it has to be passed over; but in general, irregularities due to uneven distribution have not proved as great as might have been expected. Duplicate smears have shown a surprisingly good agreement in count.

The second limitation — difficulty in recognizing bacteria — is especially great in the case of soil, not only because of the many objects in soil that closely resemble bacteria, but also because of the minute size of many soil microörganisms. Such tiny bacteria are easily overlooked, and the constant fear of overlooking them sometimes induces the investigator to count all rod-shaped bodies, many of which are probably soil particles that owe their dark appearance not to the stain but to their high refractive power. Errors arising from this cause can be avoided only by training the eye to recognize the bacteria, and there is no way to determine how great the error is.

Under such circumstances accurate counts are not possible. The counts given in this bulletin must be understood as representing very rough estimates as to the actual number of bacteria present. The best use of the method is for qualitative rather than for quantitative purposes, that is, to show what kinds of microörganisms exist in soil in active form.

In qualitative work.— When using the method for qualitative purposes, these limitations are of less importance. In this work the chief difficulty arises from the fact it is impossible to tell one kind of bacteria from another and is sometimes hard to tell to what group of microörganisms a given individual belongs. The " conidia " of Actinomycetes, for instance, are so nearly like bacteria of the short rod type that much experience is required in order to decide which is which, and even a trained eye cannot tell them apart with any degree of confidence.

In brief, the chief limitation of the method in qualitative work is the fact that the organisms observed cannot be isolated and studied in pure culture. In an attempt to overcome this limitation in part, a study is being made of various soil organisms inoculated into sterilized soil in order to obtain familiarity with their appearance under such conditions. It is hoped that the experience thus gained may make the method of more use eventually for qualitative work than it is at present.

In spite of these limitations, much interesting information has already been secured by means of the technique. The following pages will show some of the results thus far obtained, and may perhaps suggest other uses to which it may be put in the future.

RESULTS OBTAINED.

Actual number of bacteria in soil.[9]— For a long time bacteriologists have been speculating as to the real number of bacteria in soil. It has been known that many soil bacteria are unable to grow on the culture media used in plating soil and that therefore the plate counts must be lower than the true number. The question has been whether the organisms not included in plate counts are of slight numerical importance or constitute a large part of the soil flora. Improvements in the plating technic have resulted in higher counts up to a certain limit; but there seems to be a point above which counts cannot be obtained by the plate method, whatever technic be used. The only method used in the past to check up plate counts of soil bacteria is the dilution method proposed by Hiltner and Störmer.[10] This method was used by Löhnis[11] in comparison with the plate method. Löhnis obtained higher counts by the dilution method, but no higher than are often obtained today by the plate method. In fact, Löhnis' plate counts are so low as to suggest that his plating technic was unsatisfactory. The dilution method, in short, has never been shown to give strikingly higher counts than the plate method. Hence the microscopic technic is the best method yet proposed for determining the actual number of bacteria in soil.

For the reasons above discussed, the microscope cannot answer this question with as much assurance as might be desired. It has been found possible, nevertheless, to obtain interesting information on the subject.

In Table I are listed eleven comparisons between the plate and microscopic counts of field soil. It will be seen that the microscopic counts are 10 to 70 times as great as the corresponding plate counts. If the microscopic counts actually represent the true number of bacteria, there are three ways of accounting for this great discrepancy: (1) Many of the bacteria may be dead; (2) the clumps

[9] The work reported in this section of the paper was done in collaboration with J. Bright. Mention of it has already been made in a paper presented at the 1917 meeting of the Society of American Bacteriologists, entitled: What is the actual number of bacteria in soil? J. Bright and H. J. Conn. (See the proceedings of the society in the February number of *Abstracts of Bacteriology*, Vol. 2.)

[10] Hiltner, L., and Störmer, K. Studien über die Bakterienflora des Ackerbodens, mit besonderer Berücksichtigung ihres Verhaltens nach einer Behandlung mit Schwefelkohlenstoff und nach Brache. *Kaiserl. Gesundheitsamte, Biol. Abt. Land- u. Forstw.*, 3:445–545. 1903.

[11] Löhnis, F. Zur Methodik der bakteriologischen Bodenuntersuchung II. *Centbl. Bakt.* Abt. II, 14:1–9. 1905.

or masses in which they occur may not break up when plated and hence may form but one colony each; (3) many of them may be species that do not grow on the media used in plating. Altho some dead bacteria are likely to be counted, the first of these explanations is unsatisfactory, Winslow and Wilcomb[12] having shown that dead bacteria, in the presence of live organisms, rapidly lose their staining power. The second explanation is shown to be unsatisfactory by the figures given in the next to the last column of Table I. The "group counts" listed in this column were obtained by counting each clump of individuals as well as each isolated individual as a unit. If every organism seen were capable of growing on the plates this "group count" should be the same as the plate count, or even somewhat lower because of the partial breaking up of the clumps

TABLE I.—NUMBER OF BACTERIA IN NORMAL FIELD SOIL.

(Figures indicate numbers per gram of soil.)

Date.	Soil.	Plate Count.		Microscopic Count.	
		Gelatin* colonies.	Agar* colonies.	"Groups."†	Individuals.
1917.					
May 15	Dunkirk silty clay loam. Cultivated	‡17,500,000	23,000,000	140,000,000	175,000,000
July 20	Dunkirk silty clay loam. Sod.....	14,000,000	17,000,000	218,000,000	340,000,000
July 20	Dunkirk silty clay loam.. Cultivated	13,500,000	16,000,000	126,000,000	214,000,000
Aug. 10	Volusia silt loam. Sod.....	3,500,000	6,500,000	95,000,000	143,000,000
Aug. 10	Volusia silt loam. Cultivated.....	3,800,000	8,500,000	97,000,000	144,000,000
Nov. 14	Ontario fine sandy loam. Sod.....	13,500,000	18,000,000	248,000,000	390,000,000
Nov. 14	Ontario fine sandy loam. Cultivated	16,800,000	25,000,000	150,000,000	250,000,000
Nov. 21	Dunkirk silty clay loam. Cultivated	5,500,000	11,000,000	256,000,000	360,000,000
Nov. 21	Dunkirk silty clay loam. Cultivated	11,000,000	24,000,000	260,000,000	405,000,000
Nov. 23	Volusia silt loam. Sod.....	9,300,000	9,500,000	138,000,000	178,000,000
Nov. 23	Volusia silt loam. Cultivated.....	5,800,000	5,000,000	174,000,000	230,000,000

* Asparaginate-glycerin agar and tap-water gelatin were used; see Tech. Bul. 57, p. 25.
† Every isolated individual, as well as each group of two or more, was counted as a "group."
‡ This gelatin count is inexact because of rapid liquefaction.

in the process of dilution preliminary to plating. As a matter of fact, however, the "group count" is from 8 to 45 times as large as the plate count. The average group is one of only about 1.5 individuals, as shown by dividing the figures in the last column of the table by those in the next to the last. Hence the second explanation is unsatisfactory. This leaves no explanation except that the majority of the soil bacteria do not grow on the plates. This is practically the same conclusion as reached by Winslow and Willcomb in regard to sewage; while on the contrary, Brew and Dotterrer[13] have found that in the case of milk the microscopic count is greater than the

[12] Winslow, C.-E. A., and Willcomb, G. E. Tests of a method for the direct microscopical enumeration of bacteria. *Jour. Inf. Dis.*, Suppl. 1:273–283. 1905.

[13] Brew, J. D., and Dotterrer, W. D. The number of bacteria in milk. N. Y. Agr. Exp. Sta. Bul. 439. 1917.

plate count, not so much because of organisms that do not grow, as because of clumps that do not entirely break up upon plating.

This explanation holds only on the assumption that the microscopic count is actually a count of bacteria and not one of mere specks that stain like bacteria. There are, however, so many tiny objects in soil closely resembling bacteria that there is always a doubt in the mind of the investigator whether he is actually counting microorganisms. Information in regard to this point has been obtained by correlating plate and microscopic counts. In Table I it will be noticed that the highest microscopic count (test No. 9) was made from the sample with the next to the highest agar count; and the two lowest microscopic counts (Nos. 4 and 5) from the samples with the two lowest gelatin counts. It is also to be noticed that there is a tendency for the microscopic counts of Volusia silt loam to be lower than those of the other soils — a fact which agrees not only with the plate counts listed in the table but also with other plate counts of these soils and with observations as to the rate of decomposition in Volusia silt loam, all of which indicate that this soil type is of low bacterial content. It is possible, however, to pick out so many individual cases in Table I where no such correlation exists that very little light can be obtained from it as to the meaning of the microscopic count.

Further information as to the significance of the microscopic count can be obtained from the experiment summarized in Table II. In this experiment 100 grams of horse manure were mixed with 2,000 grams of soil (Dunkirk silty clay loam) and kept in the laboratory in two pots. At frequent intervals, enough water was added to restore that lost by evaporation. Care was taken to see that the soil was brought to its original moisture content about twenty-four hours before any sample was taken, so that all the samples should represent comparable moisture conditions. One pot was sampled very frequently during the first two weeks of the experiment, the other only at longer intervals.

As in the case of field soil, the microscopic counts from these pots are much higher than the plate counts. The most interesting point, however, is that both plate and microscopic counts increased for about a week after adding the manure and then decreased with more or less regularity. The maximum plate count and maximum microscopic count both occurred on the same day, six days after adding the manure. Samples of Pot 2 were not taken often enough to follow the entire rise and fall of the bacteria, but the eight counts made show the same tendency.

This correlation between the two counts suggests very strongly that the microscopic count is actually an enumeration of the bacteria. A careful scrutiny, moreover, of those counts where there is no such correlation tends to indicate a superiority of the microscopic method. It will be noticed that the fifth, sixth and seventh microscopic counts of Pot 2 are lower than the corresponding counts of Pot 1, altho no

Table II.—Number of Bacteria in Manured Soil.

(Figures indicate numbers per gram of soil.)

Time Since Adding Manure.	Pot 1.				Pot 2.			
	Plate count.		Microscopic count.		Plate count.		Microscopic count.	
	Gelatin* colonies.	Agar* colonies.	"Groups."†	Individuals.	Gelatin* colonies.	Agar* colonies.	"Groups."†	Individuals.
0 days	27,000,000	35,000,000	200,000,000	295,000,000	27,000,000	35,000,000	200,000,000	295,000,000
1 day	90,000,000	120,000,000	385,000,000	490,000,000	.....	.....	.....	.....
2 days	60,000,000	100,000,000	420,000,000	780,000,000	.....	.....	.....	.....
3 days	‡80,000,000	145,000,000	520,000,000	790,000,000	60,000,000	135,000,000	470,000,000	750,000,000
4 days	125,000,000	150,000,000	376,000,000	680,000,000	.....	.....	.....	.....
6 days	235,000,000	120,000,000	555,000,000	865,000,000	.....	.....	.....	.....
9 days	45,000,000	70,000,000	500,000,000	795,000,000	.....	.....	.....	.....
13 days	43,000,000	40,000,000	475,000,000	650,000,000	30,000,000	47,000,000	500,000,000	680,000,000
16 days	‡35,000,000	70,000,000	409,000,000	533,000,000	.....	.....	.....	.....
21 days	50,000,000	55,000,000	346,000,000	463,000,000	.....	.....	.....	.....
24 days	55,000,000	65,000,000	346,000,000	496,000,000	55,000,000	70,000,000	298,000,000	488,000,000
29 days	85,000,000	80,000,000	281,000,000	462,000,000	.....	.....	.....	.....
38 days	45,000,000	40,000,000	316,000,000	483,000,000	.....	.....	.....	.....
2 months	95,000,000	75,000,000	410,000,000	544,000,000	105,000,000	75,000,000	301,000,000	393,000,000
3 months	18,000,000	70,000,000	408,000,000	739,000,000	14,000,000	50,000,000	222,000,000	395,000,000
3½ months	.....	42,000,000	286,000,000	500,000,000	.....	46,000,000	161,000,000	291,000,000
4 months	20,000,000	23,000,000	190,000,000	445,000,000	28,000,000	45,000,000	244,000,000	506,000,000

* Asparaginate-glycerin agar and tap-water gelatin were used; see Tech. Bul. 57, p. 25.
† Every isolated individual, as well as each clump of two or more, was counted as a "group."
‡ These gelatin counts are inexact because of rapid liquefaction.

such difference is brought out by the plate method. The occurrence of this difference in three successive samples is enough to suggest that it is a real difference overlooked by the plate method. It will also be noticed that the plate count of both pots returns to its original level by the third month and the microscopic "group count" by the middle of the third month, while the microscopic count of individuals (except for the next to the last sample from Pot 2) does not reach its original level even by the fourth month. This indicates that in the manured soil some kind or kinds of organisms developed that formed clumps instead of breaking apart into isolated individuals and therefore did not affect the plate count. Similar evidence has been obtained from earlier work on manured soil which is not published in detail as it was done before perfecting the microscopic technic and the results are therefore inexact. Facts of this sort cannot be brought out by the plate method.

Further information as to the significance of the microscopic count can be obtained from the data given in Table III. In this experiment soil was placed in small Erlenmeyer flasks and sterilized two hours in an autoclave at fifteen pounds pressure, a treatment which not only killed the bacteria but disintegrated nearly all of them. Three pure cultures of common soil organisms were used for inoculating, one-third of the flasks being inoculated with each. At different lengths of time after inoculation, plate and microscopic counts were made as listed in the table. It will be noticed that the microscopic counts of *B. cereus* are always higher than the plate counts; but that the discrepancy between these two counts is much smaller than in the case of unsterilized soil. *B. cereus* is a large, easily visible organism that grows well on the plates, so fairly correct counts of it might be expected with either plate or microscope; and the relation between the two counts given in the table is much what might be anticipated. The other two organisms also grow well on bacteriological media, but they are both small bacteria, easily overlooked under the microscope. Accordingly, for both of these cultures, the plate count is considerably higher than the microscopic count — a relation the reverse of that which holds in unsterilized soil. These figures indicate that if an organism is present in soil that can grow well on the plates, the plate count will be nearly as high or even higher than the microscopic count. The logical conclusion is that the great difference between plate and microscopic counts observed in normal soil is due to organisms unable to grow on the plates.

In connection with this evidence from the pure cultures, however, one possibility must be recognized. Small particles of organic matter may be present in normal soil that take the stain and appear as bacteria under the microscope, but which lose their staining power when the soil is sterilized. If true, this might account for

the discrepancy between the two counts in normal soil which is not observed in the case of pure cultures; but it seems so unlikely that heating should affect the staining powers of dead organic matter, that no very great weight is attached to this possibility.

TABLE III.—NUMBER OF BACTERIA IN STERILIZED SOIL REINOCULATED WITH PURE CULTURES.

(Figures indicate numbers per gram of soil.)

DAYS SINCE INOCULATION.	PLATE COUNT.*	MICROSCOPIC COUNT.	
		"Groups."†	Individuals.
Culture No. 1 (*B. cereus*).			
TEST NO. 1.			
3 days	3,000,000	5,000,000	9,000,000
5 days	1,750,000	6,000,000	6,000,000
7 days	15,000,000	36,000,000	50,000,000
TEST NO. 2.			
2 days	4,000,000	20,000,000	46,000,000
4 days	5,000,000	14,000,000	27,000,000
8 days	10,500,000	18,500,000	35,000,000
10 days	10,000,000	17,000,000	25,000,000
Culture No. 2 (*Ps. fluorescens*).			
TEST NO. 1.			
3 days	260,000,000	197,000,000	204,000,000
5 days	185,000,000	133,000,000	152,000,000
7 days	475,000,000	259,000,000	325,000,000
TEST NO. 2.			
2 days	140,000,000	79,000,000	88,000,000
4 days	260,000,000	173,000,000	188,000,000
8 days	315,000,000	236,000,000	247,000,000
10 days	350,000,000	310,000,000	328,000,000
Culture No. 3. (Orange liquefying type. See Tech. Bul. 59, p. 8.)			
TEST NO. 1.			
3 days	665,000,000	492,000,000	608,000,000
5 days	4,800,000,000	1,340,000,000	1,615,000,000
7 days	1,720,000,000	1,105,000,000	1,254,000,000
TEST NO. 2.			
2 days	1,050,000,000	728,000,000	728,000,000
4 days	1,350,000,000	760,000,000	784,000,000
8 days	1,460,000,000	736,000,000	800,000,000
10 days	1,200,000,000	795,000,000	822,000,000

* Tap-water gelatin was used; see Tech. Bul. 57, p. 25.

† Every isolated individual, as well as each group of two or more, was counted as a "group."

Summing up, it may be said that altho none of these lines of evidence furnishes conclusive proof, they all tend to indicate that the microscopic count is an actual count of the living microörganisms in soil. Such being the case, it is plain that there are many more of the soil bacteria than of the milk bacteria that do not grow on the plates — just how many it is impossible to state. Probably the actual number of bacteria in soil is five, ten, or even twenty times greater than indicated by the plate count.

Spore-forming bacteria in soil.— Aside from the quantitative results, other findings of value have resulted from the work. Of special interest are those relating to the spore-forming bacteria. In previous papers [14] the significance of this group of bacteria has been discussed, and it has been shown that in spite of former ideas as to their importance, no conclusive evidence as to their activity in normal soil has yet been obtained. This conclusion was reached by means of cultural studies, unsupported by a microscopic investigation; but as soon as the microscopic technic was perfected, it was used to throw additional light on this same question.

The spore-forming bacteria are as a rule quite large, the three forms isolated most abundantly from soil (*B. megatherium* De Bary, *B. cereus* Frankland and *B. mycoides* Flügge) all having a diameter of one micron or over. They are, in fact, almost if not absolutely the only large rods that can be obtained from soil by means of culture plates. When a culture of one of these organisms is mixed with soil, its vegetative rods and spores both show very plainly under the microscope. In fact Table III shows that microscopic counts of *B. cereus* in soil were higher than the corresponding plate counts. This indicates that spore-forming bacteria could hardly have been overlooked in the soils studied by means of the microscope, and the results therefore have considerable significance.

The results are given in Table IV. In the first column of figures are listed the numbers of small rods observed (ordinarily under 0.5 micron in diameter). These counts probably include as many Actinomyces conidia as true bacteria; but the two are not listed separately because of the difficulty in distinguishing between them in all cases. The next column contains the numbers of medium-sized rods observed (about 0.5–0.8 micron in diameter). This is about the size of *Ps. fluorescens* (Flügge) Migula, and is much smaller than such forms as *B. cereus*. An occasional spore-former— *B. mesentericus* (Flügge) Migula, for instance — may be as small as this; but as these small spore-formers have rarely been isolated from soil, these figures are probably counts of non-spore-forming bacteria. The last column lists the number of spores observed.

[14] Conn, H. J. Are spore-forming bacteria of any significance in soil under normal conditions? N. Y. Agr. Exp. Sta., Tech. Bul. 51. 1916. *Jour. Bact.*, 1:187–195. 1916.

Conn, H. J. Spore-forming bacteria in soil. N. Y. Agr. Exp. Sta., Tech. Bul. 58. 1917.

In making these counts of spores, every object bearing any resemblance to a spore was counted, even tho it was suspected to be something entirely different; nevertheless the number of spores recorded was only about 1 per ct. of the total flora observed. Large rods (1 micron or more in diameter) such as those of *B. cereus*, *B. megatherium* or *B. mycoides*, were not found at all in the samples. It seems reasonable, therefore, to conclude that the large spore-formers were not active in these soils, that the small spore-formers probably were not active; but, if so, certainly comprised but a very small percentage of the total bacterial flora. This entirely corroborates the previous conclusions derived from cultural studies alone.

TABLE IV.— CLASSIFICATION BY MEANS OF THE MICROSCOPE OF THE BACTERIA IN NORMAL FIELD SOIL.

Date.	Soil.	Bacteria per Gram, Determined Microscopically.		
		Small rods.	Medium-sized rods.	Spores.
1917.				
May 15	Dunkirk silty clay loam. Cultivated....	167,000,000	3,000,000	5,000,000
July 20	Dunkirk silty clay loam. Sod.....	334,000,000	3,000,000	3,000,000
July 20	Dunkirk silty clay loam. Cultivated....	206,000,000	4,000,000	4,000,000
Aug. 10	Volusia silt loam. Sod.....	141,000,000	1,500,000	500,000
Aug. 10	Volusia silt loam. Cultivated.....	140,000,000	3,500,000	1,000,000
Nov. 14	Ontario fine sandy loam. Sod.....	375,000,000	12,000,000	3,000,000
Nov. 14	Ontario fine sandy loam. Cultivated....	245,000,000	3,000,000	3,000,000
Nov. 21	Dunkirk silty clay loam. Cultivated....	350,000,000	8,000,000	3,000,000
Nov. 21	Dunkirk silty clay loam. Cultivated....	390,000,000	7,000,000	6,000,000
Nov. 23	Volusia silt loam. Sod.....	172,000,000	4,000,000	2,000,000
Nov. 23	Volusia silt loam. Cultivated.....	228,000,000	1,500,000	750,000

Absence of mold hyphae.— One of the most surprising results so far obtained is to find that mold hyphae occur so seldom in the soils studied that for all practical purposes they may be considered absent. Attention has already been called to this point.[15] They are not only too rare to be included in any of the counts given above, but actually occur so seldom in many soils that hundreds of microscopic fields can be examined without finding a single fragment of mycelium. There is, of course, no question but that fungi can live in soil; and such an unexpected finding gave rise to the suspicion that fungi might assume some unfamiliar form when growing in soil and might therefore escape recognition. To check up this point, some woodland soil, with more or less leaf mold on the surface, was examined by the usual technic. In this preparation mold hyphae were found, not by any means as abundantly as the bacteria but rather more so than Actinomyces filaments. This shows quite plainly that the technic is capable of revealing fungus mycelium

[15] Conn, H. J. Relative importance of fungi and bacteria in soil. *Sci.*, N. S., 44:857–858. 1916.

when present; and the failure to find it in any microscopic preparation presumably means that fungus growth is not sufficiently abundant in that particular soil to show under the microscope.

Considering that Waksman [16] has recently reported finding considerable numbers of fungi in soils obtained from all parts of the country, it seemed surprising, at first thought, that they were not abundant enough in any of the agricultural soils studied to be revealed by the microscope. It was found, however, by microscopic examination of a colony of Aspergillus growing on an agar plate, that this colony bore spores enough to give a plate count of 300,000 per gram (about the average plate count of fungi found by Waksman) provided it were distributed evenly thruout a kilo of soil and every spore were capable of growth on culture plates; and yet such a small amount of mycelium would be added to the soil that only one fragment of mold hyphae would be found in every 3,000 microscopic fields, using the dilution and magnification employed in the present work. This suggests that if a fungus capable of producing spores in the soil were abundant enough to show mycelium under the microscope, its plate count would be enormous. It shows further that a soil might have a plate count of fungi as high as reported by Waksman without showing mycelium under the microscope. In other words, there is no real inconsistency between the present findings and those of Waksman.

If in comparing Waksman's conclusions with the results of the present investigation, the question arises as to whether the plate count or the microscopic study has the greater significance, there can be but one answer. It must be admitted that plate counts of organisms like Actinomycetes and higher fungi do not have the same significance that plate counts of bacteria do. If in the kilo of hypothetical soil above mentioned just ten spores should germinate and produce colonies bearing as many spores as the original colony, the plate count would become 3,300,000 per gram without any very great fungus activity taking place. Suppose, on the other hand, this mold were not capable of spore-production in soil. Then it might grow vigorously and profoundly modify the character of the soil while the plate count, which would still indicate the number of spores, would probably diminish. Obviously the plate count of a fungus indicates, not numbers of individuals, but ability to produce spores.

It should further be pointed out that the species of fungi found most abundantly by Waksman (Mucorales, Aspergilli, Penicillia, etc.) are common dust fungi; and their general occurrence, therefore, in soils from different parts of North America and Europe does not prove that soil has a characteristic fungus flora. On the other hand, it is not claimed that the failure to find fungi in microscopic

[16] Waksman, S. A. Is there any fungus flora of the soil? *Soil Sci.*, 3:565–589. 1917.

preparations disproves their activity in agricultural soils in general. The matter is discussed here in order to explain the apparent inconsistency between the results obtained by the plate method and those by the microscopic method; and to emphasize need of caution in interpreting the plate count of a spore-forming organism.

Actinomycetes in soil.— In an earlier paper [17] the question was raised whether Actinomycetes occur in soil as filaments or as "conidia." The microscope has partly answered this question. Both forms occur. The "conidia," however, are so much more numerous than the filaments that there is no question but that practically all the colonies of Actinomycetes on the plates arise from spores. Just as in the case of higher fungi, the plate count is not an index of activity but of ability to produce spores. The use of the microscope is necessary to interpret the plate count. Microscopic examination shows the great preponderance of spores over filaments and proves that a very high plate count of Actinomycetes does not necessarily indicate more than a slight activity of these organisms. Their filaments, however, are not lacking in any soil yet studied; and occasionally large colonies have been found, containing both filaments and conidia. Altho such masses of actively growing Actinomycetes are quite rare compared to the numbers of true bacteria observed, they are much more common in ordinary soil than are the hyphae of higher fungi. This indicates that Actinomycetes are comparatively active in soil, altho they are probably not as important as the plate count would suggest.

Arrangement of the soil bacteria.— Plate cultures give no idea whether the colonies arise from single organisms or from clumps containing thousands. Brew and Dotterrer [18] have already shown that the average clump of bacteria in market milk contains between eight and eleven individuals; but in the soils so far studied the clumps of bacteria are much smaller. A great majority of the soil bacteria occur singly. A striking exception is a certain small short rod found quite abundantly in some samples of the surface crust of soil. This rod occurs in large sheets one individual thick, but sometimes containing thousands of individuals. The rods are arranged in rows, curved or straight, that form beautiful patterns. Sometimes they have been observed forming sheets around mineral particles; and at other times they have been found in hollow masses, irregularly spherical and always somewhat torn, which there is good reason to believe were originally formed around mineral particles, the soil particle having been forced out during the process of making the smear. The identity of this organism is still unknown; and it may be important in soil, altho it is not likely to occur to a large extent upon plates made from soil.

[17] Conn, H. J. Actinomycetes in Soil. N. Y. Agr. Exp. Sta., Tech. Bul. 60. 1917.
[18] See footnote 11.

The size of the average group of bacteria in soil is important in interpreting the plate count. As the bacteria ordinarily occur singly or in small groups, the plate count is probably a fairly accurate count of those bacteria capable of growing on the plates. The discrepancies between plate and microscopic counts are due not to the breaking up of clumps but to the inability of many of the organisms to grow on the plates.

CONCLUSIONS.

As a result of this study, the conclusion seems justified that the microscope offers very promising possibilities for the study of bacteria and fungi in soil. Alone it does not yield results of as great value as when used in conjunction with cultural methods; but in connection with them it can furnish direct evidence in regard to many obscure points. Its principal uses seem to be to determine the number of bacteria in soil that do not grow on culture plates, and to learn whether an organism with spores or resting-stage is active or inactive in any particular soil. The data along these lines presented in the present bulletin are not very extensive, but it is hoped that they will suggest to other workers further uses to which the method may be put.